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Stress Induction of the Virulence Proteins (SpvA, -B, and -C) from Native Plasmid pSDL2 of *Salmonella dublin*

S. E. VALONE,[1,2]* G. K. CHIKAMI,[1]† AND V. L. MILLER[2]

Division of Infectious Diseases, Department of Medicine, Center for the Health Sciences 37-121, University of California, Los Angeles, California 90024-1736,[1] and Department of Microbiology and Molecular Genetics, University of California, 405 Hilgard Avenue, Los Angeles, California 90024-1489[2]

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The virulence region of the wild-type plasmid pSDL2 contained in *Salmonella dublin* is highly conserved among plasmids from several nontyphoid *Salmonella* serotypes and is essential for the development of systemic infection in BALB/c mice. Polyclonal antibodies against three proteins (SpvA, -B, and -C) expressed from a 4.1-kb EcoRI subclone of the plasmid virulence region were generated. These antibodies were used to detect expression of the Spv proteins when *S. dublin* was grown in vitro under stress-inducing conditions, such as nutrient deprivation and increased temperature, that the bacteria may encounter during the course of infection within the host. Glucose starvation resulted in expression of all three proteins shortly after the lag phase. When the bacteria were grown to the late-log phase without glucose, heat shock strongly induced expression of SpvA but not SpvB or SpvC. The addition of 0.2% glucose to the medium resulted in loss of expression of the proteins until the late-log to stationary phase. Iron limitation or lowered pH induced expression of the proteins during exponential growth even in the presence of glucose. Insertion mutations into the positive regulator gene *spvR* upstream from *spvABC* and insertions into *spvA* and *spvC* resulted in loss of expression of SpvA, -B, and -C, suggesting a complex regulation of expression. These studies define a variety of environmental conditions that induce expression of the Spv virulence proteins from the wild-type plasmid pSDL2 in *S. dublin* in vitro.

Salmonella dublin is host adapted to cattle and causes significant morbidity and mortality when herds of cattle are infected endemically. Adult cattle infected with *S. dublin* can suffer from abortion and chronic mastitis, and calves can develop septicemia resulting in death (45). In humans, infection with certain *Salmonella* serotypes, including *S. dublin*, is associated with an increased predilection for the development of systemic infection that can lead to death (3, 53).

The strain of *S. dublin* that was used in this work (*S. dublin* Lane) was isolated from the blood of a patient; like most clinical isolates of *S. dublin*, the Lane strain contains an 80-kb plasmid designated pSDL2 (22). The presence of this virulence plasmid in nontyphoid salmonellae has been associated with the development of systemic illness. Fierer et al. reported that 71% of *Salmonella typhimurium* isolates obtained from the blood of patients contained the virulence plasmid, whereas only 42% of fecal isolates contained the plasmid (13). Heffernan et al. (22) compared the clinical course of BALB/c mice orally infected with the plasmid-containing *S. dublin* Lane strain and a plasmid-free derivative, LD842. LD842 was able to colonize the small bowel, multiply in the Peyer's patches, and spread to the liver and spleen, but the bacteria were eventually eliminated from the mice with the formation of granulomata. Infection with the Lane strain resulted in microabscess formation after rapid multiplication of the bacteria in the liver and spleen and culminated with overwhelming sepsis and death (22). The essential role of the plasmid in the expression of virulence was definitively established when Chikami et al. demonstrated that reintroduction of the plasmid into the plasmid-free strain LD842 restored the virulent phenotype in BALB/c mice (6).

Subsequent work with deletion derivatives and transposon mutagenesis of pSDL2 revealed that an 8.2-kb *Xho*I-*Sal*I fragment was sufficient for the full expression of virulence in BALB/c mice (26). Williamson et al. obtained similar results with a different strain of *S. dublin* (54). Analysis of the nucleotide sequence of this 8.2-kb plasmid region from several *Salmonella* serotypes revealed six highly conserved open reading frames in the same orientation (17, 18, 26, 30, 31, 36–38, 40, 47, 49). The different nomenclatures assigned to the genes encoding these putative proteins were recently summarized by Krause et al. (25). The *spvR*, *spvABCD*, and *orfE* designations of Caldwell and Gulig (5) were adapted for this paper. Three proteins with predicted molecular masses of 28.1, 65.6, and 27.6 kDa (SpvA, -B, and -C, respectively) are expressed from a 4.1-kb *Eco*RI fragment internal to the 8.2-kb *Xho*I-*Sal*I fragment and were studied in this work. Using this 4.1-kb *Eco*RI subclone for DNA hybridization studies, Roudier et al. demonstrated a high degree of homology among the plasmids from several different *Salmonella* serotypes, including *S. typhimurium*, *S. enteritidis*, *S. choleraesuis*, and *S. gallinarum* (42). The nucleotide sequences of *spvABC* revealed no significant similarity to any previously reported genes and thus did not provide any clues about the function of their protein products. *spvR*, the gene upstream from *spvA*, did show some similarity to a group of bacterial transcriptional activator genes (17, 26, 36, 40, 48); the region of homology is restricted to the N terminus of the predicted protein sequence, which contains the putative helix-turn-helix DNA binding motif.

Although very little is known about the function of the Spv virulence proteins, a number of studies have begun to provide information concerning their regulation of expression. Northern blot analysis of RNA prepared from salmonellae expressing the *spv* genes revealed mono- and polycis-

* Corresponding author.
† Present address: Food and Drug Administration, Rockville, MD 20857.

tronic RNA transcripts originating upstream of *spvA*, suggesting a complex regulation of expression (25). Using *lac* promoters to overexpress *spvR* or high-copy-number plasmid vectors containing *spvR*, a number of groups recently demonstrated the role of SpvR as a positive regulator of downstream *spv* genes (5, 9, 25, 29, 50). Detection of the Spv proteins has been accomplished previously only through the use of cloned genes or expression systems (17, 24, 35, 41, 46, 47); expression of the Spv proteins from the native plasmids has never been reported.

From the time of entry into the gastrointestinal tract to phagocytosis by macrophages, salmonellae encounter a variety of host defense mechanisms that subject the bacteria to harsh local environmental conditions. Virulence factors expressed by pathogenic bacteria often allow the organisms to survive hostile conditions such as increased temperature, decreased oxygen tension, acidic pH, and nutrient deprivation. By examining some of the environmental states encountered by salmonellae during the course of infection, we identified a number of conditions that resulted in the expression of the Spv plasmid virulence proteins from wild-type *S. dublin* in vitro.

MATERIALS AND METHODS

Bacterial strains and plasmid constructs. Bacteria were maintained at −70°C in Luria-Bertani (LB) broth containing 40% glycerol (vol/vol) or on LB–1.5% agar plates with appropriate antibiotic selection. Antibiotics were added for selection at 200 μg/ml for penicillin and 50 μg/ml for kanamycin. The virulent, plasmid-containing *S. dublin* Lane strain was isolated from the blood of a patient at the Veterans Administration Medical Center, San Diego, Calif., and *S. dublin* LD842 is an avirulent, isogenic, plasmid-free strain derived from the Lane strain. Both the Lane and LD842 strains are auxotrophs for nicotinamide and have been described previously (6). *Escherichia coli* JA221 (*leuB* Δ*trpE5 lacY recA hsdM*$^+$) was described previously (16). pSDL2 (80 kb) is the wild-type plasmid of *S. dublin* Lane, and p9-18Δ7 (37.4 kb) is a deletion derivative of pSDL2 containing all the necessary replication and virulence functions as well as a Tn*5-oriT* insert (Km^r) (6). The Tn*1725* insertions into p9-18Δ7 that were used in this work were described previously (8). The oligonucleotide mutations (a kind gift from D. Guiney) were constructed by insertion of translation termination linkers into pCR4, a plasmid containing the 8.2-kb *Xho*I-*Sal*I virulence region of pSDL2 and includes genes *spvR*, *spvA* through *-D*, and *orfE* (22). pEL11 was constructed by subcloning the 4.1-kb *Eco*RI fragment from the virulence region of pSDL2 in pMMB66EH (51). pMMB66EH contains the *tac* promoter, the *lacI*^{q} repressor, and a β-lactamase gene (15).

Partial purification of the virulence proteins and generation of polyclonal rabbit antibodies. *E. coli* JA221 carrying pEL11 was grown in the presence of isopropyl-β-D-thiogalactopyranoside (IPTG) to overexpress SpvA, -B, and -C, which were under the control of the *tac* promoter (51). To obtain partially purified preparations of the virulence proteins, *E. coli*(pEL11) overexpressing the proteins was subjected to cell fractionation with Triton X-100 as described previously (39). Briefly, bacteria grown under inducing conditions were collected by centrifugation, resuspended in 10 mM *N*-2-hydroxyethylpiperizine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.5), and broken open with passage through a French press. Unlysed cells were removed by centrifugation (11,500 × *g* for 40 s). The membrane fractions were collected by an additional centrifugation (15,000 × *g* for 30 min at 4°C) and resuspended in 10 mM HEPES–2% Triton X-100. After incubation at room temperature overnight with tumbling, the outer membranes (Triton X-100-insoluble fraction) were separated from the inner membranes by centrifugation for 30 min at 4°C. The Triton X-100-insoluble fraction was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically to nitrocellulose paper (Schleicher & Schuell, Keene, N.H.). The proteins were visualized by staining the nitrocellulose paper with amido black, which reportedly does not interfere with the antigenicity of the stained proteins (28). The proteins produced from the 4.1-kb *Eco*RI fragment were identified by comparison to a single column loaded with a Triton X-100-insoluble fraction prepared from uninduced *E. coli* pEL11. The induced proteins were cut from the nitrocellulose paper, dissolved in dimethyl sulfoxide, and precipitated as a particulate suspension in an equal volume of 0.05 M carbonate-bicarbonate buffer (pH 9.6) as described previously (1). Each of these suspensions was diluted 1:1 in Freund's complete adjuvant and injected subcutaneously into individual New Zealand White female rabbits. Rabbits with preimmune serum lacking cross-reactive antibodies against the Spv proteins serum were chosen for immunization. Booster immunizations were diluted 1:1 in Freund's incomplete adjuvant and were given 20 and 31 days later. A specific antibody response for each of the antigens was tested by Western immunoblot analysis (see below) approximately 1 week after each booster injection.

Growth conditions. Induction of the virulence proteins from the wild-type plasmid was tested under the following conditions. The bacteria were grown in minimal medium supplemented with 5 mg of Casamino Acids (vitamin free; Difco Laboratories, Detroit, Mich.) per ml, 0.08 μg of nicotinamide per ml, 5 μg of thiamine per ml, and 1 mM $MgSO_4$ (complete medium). Minimal salts used included M_9 (43), M_{63} (33), 3-(*N*-morpholino)propanesulfonic acid (MOPS; Sigma) buffer [80 mM MOPS, 15 mM $(NH_4)_2SO_4$, 0.7 mM K_2HPO_4 (pH 7.4)], or 2-(*N*-morpholino)ethanesulfonic acid (MES; Sigma) buffer [80 mM MES, 15 mM $(NH_4)_2SO_4$, 0.7 mM K_2HPO_4 (pH as indicated)]. Iron and calcium conditions were tested in complete MOPS medium (pH 7.4), and the pH studies were tested in complete MES medium (pH 5.0, 5.5, and 6.0) or in complete MOPS medium (pH 6.5, 7.0, and 7.4). To vary iron conditions, complete MOPS medium was supplemented with 15 or 150 μM $FeCl_2$ or with 50 to 200 μM 2,2′-dipyridyl (Sigma) to deplete iron. To vary calcium conditions, complete MOPS medium was supplemented with 2.5 mM $CaCl_2$ or with 20 mM sodium oxalate and 20 mM $MgCl_2$ to deplete the calcium. Experiments determining the effect of temperature on expression of the proteins were carried out in complete M_9 or MOPS minimal medium, and the effect of anaerobiosis was tested in complete M_{63} minimal medium. The medium was made anaerobic by the method of Cotter and Gunsalus (7). Briefly, the medium was boiled and cooled under a stream of nitrogen and dispensed anaerobically into nitrogen-flushed tubes. Anaerobic conditions were tested with glucose supplementation only; all of the other experiments were carried out both in the absence and in the presence of 0.2% glucose.

Overnight cultures of *S. dublin* Lane and LD842 grown in complete minimal medium plus 0.2% glucose were inoculated (1:100) into fresh medium as defined above. All cultures were grown with aeration by shaking at 37°C, unless otherwise specified. The bacterial cultures from each experiment were adjusted to similar optical densities (determined

on a Beckman Du-70 spectrophotometer at an optical density at 600 nm [OD_{600}]), and the cells were collected by centrifugation. The cells were resuspended in 10 mM Tris–5 mM EDTA buffer (pH 8.0) for Western blot analysis or frozen at −20°C for future evaluation. All experiments were reproducible, and conditions that resulted in induction of the Spv proteins were repeated at least two additional times after the initial observation.

SDS-PAGE and Western blot analysis. SDS-PAGE (27) was used to separate whole-cell lysates of *E. coli* JA221 (pEL11) grown with or without induction of the virulence proteins or salmonellae grown under the various conditions described above. Equal amounts of whole-cell lysates were loaded onto each lane in each experiment by adjusting the optical densities to similar values. The proteins were transferred electrophoretically (Trans-Blot cell; Bio-Rad, Richmond, Calif.) to nitrocellulose paper (Scheicher & Schuell) with a total current of approximately 1,500 mA. The blots of the proteins from *E. coli* were stained with Ponceau S (0.5% Ponceau S in 1.0% acetic acid) to verify expression of the Spv proteins and mark their location on the blots. The Ponceau S was then washed away with double-distilled H_2O. After the Western blot was probed with the rabbit antiserum (see below), the bands could be correlated with the expected locations of the overexpressed Spv proteins.

Western blot analysis were carried out as described previously (51). The blots were incubated overnight at 4°C with pooled polyclonal rabbit serum at dilutions of 1:500 to 1:1,000 when probing for the overexpressed proteins in *E. coli* and 1:25 when probing for expression of the virulence proteins from the pSDL2 or pSDL2 derivatives of *S. dublin*. Binding of the primary antibody was detected by using goat anti-rabbit immunoglobulin G (1:10,000) conjugated to alkaline phosphatase (Cappel, West Chester, Pa.) and then incubation in a reaction mixture for alkaline phosphatase containing 0.5% (wt/vol) 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (Sigma) and 0.1% (wt/vol) *p*-nitroblue tetrazolium (Sigma) in 50 mM Tris–3 mM $MgCl_2$ (pH 10).

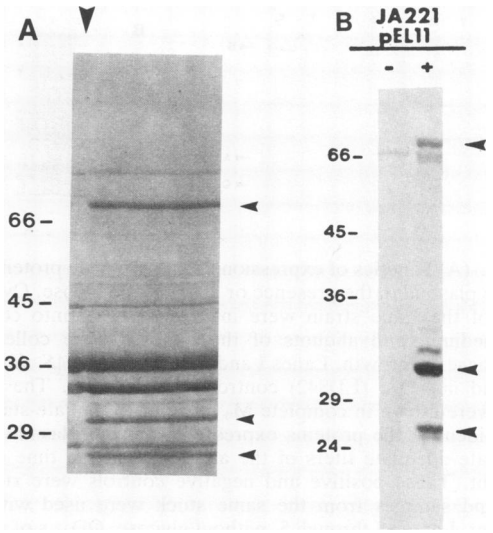

FIG. 1. (A) Electroblot of partially purified virulence proteins SpvA, -B, and -C. Whole-cell lysates were prepared from *E. coli* JA221(pEL11) that were grown in the presence of IPTG to induce production of the virulence proteins and subjected to Triton X-100 cell fractionation. The Triton X-100-insoluble fraction was separated by SDS-PAGE and transferred electrophoretically to nitrocellulose. The horizontal arrows indicate the locations of the virulence proteins. These proteins were not present when the bacteria were grown without IPTG (column with the vertical arrow at the top). (B) Western blot of *E. coli* JA221(pEL11) probed with pooled polyclonal rabbit antiserum against the virulence plasmid proteins SpvA, -B, and -C. The proteins shown in panel A were cut from the nitrocellulose and used for immunization into rabbits. Anti-SpvA (30.5 kDa), anti-SpvB (76 kDa), and anti-SpvC (27 kDa) sera were used at dilutions of 1:1,000, 1:500, and 1:1,000, respectively.

RESULTS

Partial purification of the virulence proteins and generation of polyclonal antibodies against the virulence proteins. We previously reported expression of three proteins (SpvA, -B, and -C, with apparent molecular masses of 30.5, 76, and 27 kDa, respectively) from pEL11. pEL11 contains a 4.1-kb *Eco*RI subclone from the virulence region of pSDL2, and expression of the three proteins from the subclone is under the control of the *tac* promoter (51). Triton X-100 cell fractionation of *E. coli*(pEL11) overexpressing the Spv proteins revealed that all three proteins were located in the Triton X-100-insoluble fraction (enriched for outer membrane proteins). When *E. coli*(pEL11) was grown without induction, the virulence proteins were not produced (Fig. 1A). This selective localization of the Spv proteins in the Triton X-100-insoluble fraction allowed us to obtain partially purified preparations of these proteins. There was no attempt to determine or ensure the integrity of the separation by examining enzymes characteristically identified in each cell fraction, and thus strong conclusions about the location of these proteins cannot be drawn from these studies. It is quite possible that overexpression of these Spv proteins in *E. coli* results in the formation of aggregates and their subsequent localization to the outer membrane. Other investigators have also reported that these three proteins are located in the membrane fractions when overexpressed in *E. coli* (17, 46).

These results differ somewhat from the cellular location of these proteins that we reported previously. When the Spv proteins were overexpressed in salmonellae and fractionated by sucrose gradients, SpvA was found in the outer membrane, SpvC was found in both the outer membrane fraction and the cytoplasm, and SpvB was located in the cytoplasm (51). In these studies the integrity of the separation of the various cellular fractions was examined carefully by measuring the activities of enzymes characteristically located in the cytoplasm, inner membrane, and periplasm and thus allowed conclusions about cellular location to be made (51). More definitive localization studies with salmonellae expressing the Spv proteins from the native plasmid remains to be done.

Rabbits were immunized separately with each of the partially purified proteins SpvA, -B, and -C as described in Materials and Methods. Electroblots of *E. coli* JA221 (pEL11) overexpressing the Spv proteins that were probed with immune rabbit sera, revealed the presence of antibodies against each of these proteins (Fig. 1B). A cross-reactive band at approximately 66 kDa may also be seen in the lane showing the bacteria grown under noninducing conditions, although this band is clearly distinct from the SpvB protein band. A doublet at approximately 30 (corresponding to SpvA) and 33 kDa was also noted (Fig. 1A and B). Both the 30- and 33-kDa proteins were recognized when the electroblots were probed with the SpvA antiserum alone. At times, the SpvA protein also appeared to be expressed as a doublet

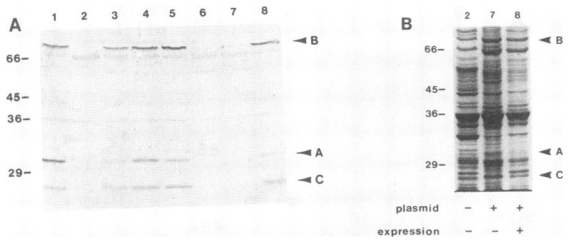

FIG. 2. (A) Kinetics of expression of the virulence proteins from the native plasmid in the presence or absence of glucose. Overnight cultures of the Lane strain were inoculated 1:100 into complete MOPS medium, and aliquots of the cultures were collected at various phases of growth. Lanes 1 and 2 show positive [*S. dublin*(p9-18Δ7)] and negative (LD842) controls, respectively. The control bacteria were grown in complete M_9 medium to the late-stationary phase to identify the proteins expressed from the plasmid and to demonstrate adequate titers of the antibodies at the time of each experiment. These positive and negative controls were stored at −20°C, and samples from the same stock were used with each experiment. Lanes: 3 through 5, without glucose, OD_{600}s of approximately 0.25, 0.65, and 1.5, respectively; 6 through 8, with 0.2% glucose, OD_{600}s of approximately 0.25, 0.65, and 2.5, respectively. Approximately equal amounts of the whole-cell lysates were loaded per lane onto 7.5% SDS–polyacrylamide gels after adjusting the OD_{600}s of the bacteria for each time point to similar values. (B) SDS-PAGE of whole-cell lysates of *S. dublin*. Bacteria shown in lanes 2, 7, and 8 correspond to their respective lanes in panel A above and were prepared from the same experiment. Coomassie blue-stained whole-cell lysates (lanes): 2, *S. dublin* LD842 (without the plasmid), grown to stationary phase; 7, *S. dublin* Lane (wild-type plasmid without expression of the Spv proteins); 8, *S. dublin* Lane (wild-type plasmid with expression of the proteins). No difference in protein expression can be seen at the expected sites of the Spv proteins.

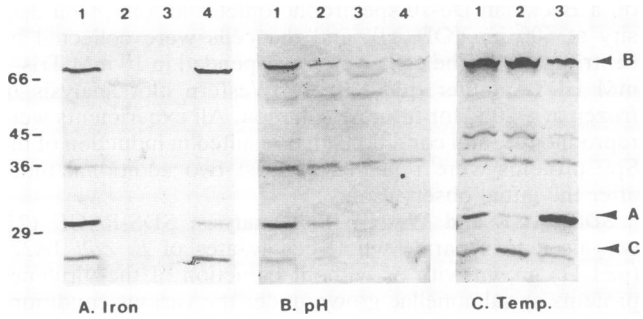

FIG. 3. Environmental conditions that induce expression of the virulence proteins from the native plasmid. Three separate experiments are shown. The Lane strain was grown to the log phase (OD_{600} of 0.6 to 0.8) in complete medium with 0.2% glucose (A and B) or without the addition of glucose (C), with the following modifications. (A) Depletion of iron. Lanes: 1 and 2, positive [*S. dublin*(p9-18Δ7)] and negative (LD842) controls, respectively, as noted for Fig. 2; 3, MOPS; 4, MOPS–50 μM dipyridyl. (B) Acidification. Lanes: 1 through 3, MES (pH 5.0, 5.5, and 6.0, respectively); 4, MOPS (pH 7.4). (C) Heat shock. Lanes: 1 through 3, M_g at 23, 37, and 42°C, respectively. Positive and negative controls as shown for panel A above were also included for the experiments shown in panels B and C and demonstrated adequate levels of antibody to detect expression of the Spv proteins (data not shown).

from the wild-type plasmid (data not shown), suggesting that this protein may be processed or degraded. We previously reported that SpvB appeared as a doublet when overexpressed in salmonellae and examined by SDS-PAGE (51), although this was not apparent when the SpvB protein was expressed from the wild-type plasmid (Fig. 2 and 3). Gulig et al. reported that all three proteins (SpvA, -B, and -C) appeared as doublets when expressed in *E. coli* minicells and evaluated by SDS-PAGE (17). The significance of these doublets remains to be determined. None of the predicted amino acid sequences of the proteins reveals the presence of classical signal sequences (26). Additionally, the N-terminal amino acid sequences of the proteins recovered from outer membranes of *E. coli* overexpressing the proteins agreed with the predicted N-terminal amino acid sequences, suggesting that no processing had occurred (46).

Induction of the plasmid virulence proteins in vitro. Elucidation of the function and regulation of the *Salmonella* plasmid virulence proteins has been hampered by their relatively low level of expression in vitro. The polyclonal antibodies described above allowed detection of the virulence proteins grown under a variety of conditions in vitro (see below). The conditions tested were those that have been shown to regulate other bacterial virulence factors as well as those that may be encountered by the organisms during the course of a natural infection.

Growth phase and glucose availability. Previous studies have suggested that the Spv proteins are expressed during the stationary phase and under conditions of glucose limitation (5, 9, 25). Similarly, our results revealed that in all media (LB, M_9, MOPS, MES) and under all conditions examined except for anaerobiosis, the Spv proteins were expressed in bacteria grown to the stationary phase. However, we also clearly found expression of SpvA, -B, and -C during exponential growth. Complete medium containing Casamino Acids (M_9 or MOPS), but not supplemented with glucose, resulted in expression of the proteins in early-log-phase growth (OD_{600} of 0.2 to 0.3) (Fig. 2A). These results indicate that cessation of growth is not a necessary condition for the induction of these proteins. When 0.2% glucose was added to the complete medium, expression of the Spv proteins was delayed until the bacteria reached the late-log to stationary phase, although low levels of SpvB were detected during exponential growth (OD_{600} of 0.6 to 0.8) (Fig. 2A). Addition of as little as 0.05% glucose resulted in loss of expression of the proteins during the early-log phase (data not shown). Positive and negative controls (*S. dublin* p9-18Δ7 and LD842, respectively, grown to the stationary phase) were included for every experiment and always demonstrated that the pooled serum (1:25) contained antibodies in adequate titers to detect expression of the SpvA, -B and -C proteins.

Lack of expression of the Spv proteins in the presence of glucose may indicate that glucose starvation is the signal for expression of these proteins in the late-stationary phase. Stationary-phase growth, however, was not always sufficient for expression of these virulence proteins. Anaerobiosis resulted in significantly slower growth of the bacteria and a much lower cell density at the stationary phase, yet the Spv proteins were never induced (data not shown). These results may suggest that oxygen is needed for induction of the Spv proteins.

Unlike some of the plasmid virulence proteins expressed from *Yersinia* spp., which are easily visible in the culture supernatant or after staining with Coomassie blue (32), expression of the Spv proteins could not be detected by inspection of Coomassie blue-stained gels of whole-cell lysates of *S. dublin*. Comparison between *S. dublin* LD842 (without the plasmid), *S. dublin* Lane (wild-type plasmid) not expressing the virulence proteins, and *S. dublin* Lane

expressing the virulence proteins revealed no obvious differences in protein band patterns at the expected location of the SpvA, -B, and -C proteins (Fig. 2B). Some differences in protein expression due to the different growth phases were noted at molecular weights other than the Spv proteins.

The differential expression of the Spv proteins during the log phase in the presence or absence of glucose allowed further evaluation of conditions that may downregulate or induce expression of the Spv proteins. Thus, the experiments described below were carried out with or without 0.2% glucose and evaluated for expression of the virulence proteins during log-phase growth (OD_{600} of 0.6 to 0.8).

Acidification and iron and calcium concentrations. Calcium plays an instrumental role in regulation of several virulence genes in *Yersinia* spp. (2, 32), but changes in calcium concentrations did not affect expression of the Spv proteins. Decreased iron availability and lowered pH are conditions that salmonellae may encounter intracellularly within macrophages. Neither of these conditions affected expression of the proteins when the bacteria were grown without glucose (data not shown). However, when stressed with iron limitation or lowered pH in the presence of glucose, *S. dublin* Lane expressed SpvA, -B, and -C during exponential growth (Fig. 3A and B). In the presence of glucose, growth of the bacteria to an OD_{600} of approximately 0.6 to 0.8 was necessary to induce the proteins by iron deprivation or acidification as compared with glucose starvation, which clearly induced expression of the proteins in the early-log phase. Depending on the batch of medium and dipyridyl used, between 50 and 200 μM dipyridyl resulted in increased expression of the proteins. Addition of excess $FeCl_2$ to medium containing dipyridyl resulted in loss of expression of the Spv proteins, indicating that decreased iron concentration was responsible for induction (data not shown).

Addition of both Casamino Acids and 0.2% glucose to the medium allowed the bacteria to reach an OD_{600} of greater than 2.0, except when the bacteria were grown at pH 5.0, when the cells reached an OD_{600} of approximately 1.9. Varying the conditions of iron or acidification did not change the bacterial growth pattern significantly (Fig. 4A).

Temperature. Upon entering the host, salmonellae encounter a change from the ambient temperature to 37°C; subsequent pyrogen production by the host results in exposure of the bacteria to even higher temperatures. Interestingly, the strongest inducer of the SpvA protein was a combination of glucose starvation and heat shock. When the bacteria were grown at 42°C without glucose to an OD_{600} of 0.6 to 0.8, the SpvA protein was expressed at levels much higher than those with growth at 37 or 23°C (Fig. 3C). Increased expression of SpvA was not seen during exponential growth when the bacteria were grown at 42°C in the presence of glucose (data not shown). These data suggest that under compounded stress SpvA expression may be part of a heat shock response but that heat shock in itself is not an adequate signal for its induction.

The addition of Casamino Acids but not glucose to the minimal medium allowed the bacteria to reach an OD_{600} of approximately 1.5. Heat shock and glucose starvation did slow bacterial growth. However, decreased growth rate alone could not explain induction of the proteins (under heat shock and glucose starvation conditions) because other conditions (iron deprivation and glucose starvation [Fig. 4B] and anaerobiosis) also reduced the growth rate without increased induction.

Expression of the *spvA*, *-B*, and *-C* genes containing insertion mutations. There is growing evidence that the *spv* genes

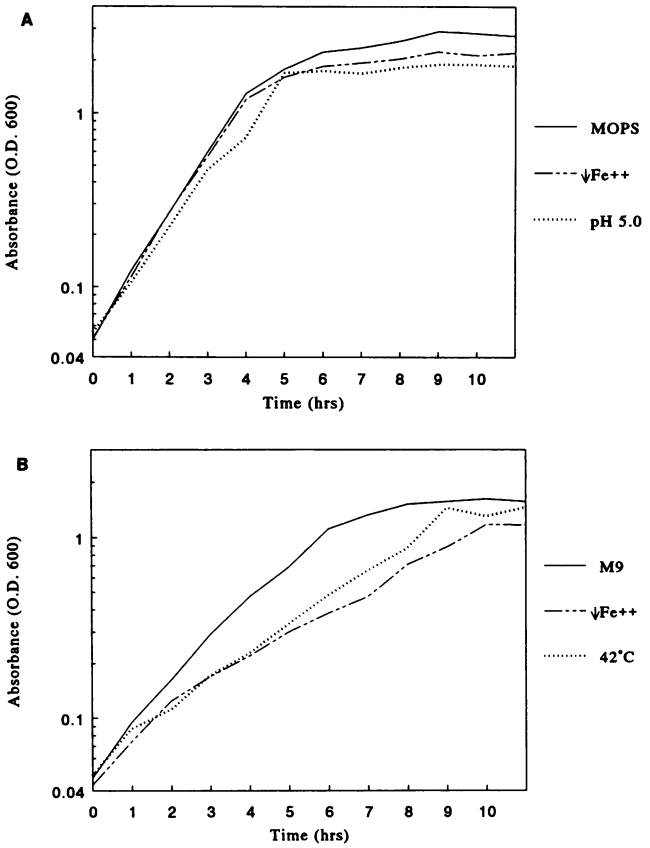

FIG. 4. Growth curves of *S. dublin* Lane under various environmental conditions. (A) Overnight cultures of *S. dublin* Lane were inoculated into complete medium plus glucose at a dilution of 1:100 and grown at 37°C with aeration, with the following modifications: (——) MOPS medium, (— — — —) MOPS medium–100 μM dipyridyl, (- - -) MES medium (pH 5.0). (B) Complete medium without glucose. Cultures of *S. dublin* Lane were prepared as described above: (——) M_9 medium, (— — — —) MOPS medium–100 μM dipyridyl, (- - -) M_9 medium, 42°C. The OD_{600}s of bacterial cultures were determined at 1-h intervals over a 12-h period.

are organized in a complex and somewhat unusual operon (25, 50). *S. dublin* carrying individual transposon insertions (p9-18Δ7::Tn*1725*) into *spvR* and *spvA -B*, and -C were studied to determine the effect of these mutations on the expression of SpvA, -B, and -C. p9-18Δ7 is a deletion derivative of the wild-type plasmid; when *S. dublin*(p19-18Δ7) was grown under inducing conditions, the levels of expression of the Spv proteins were near wild-type levels (Fig. 5, lanes 2 and 3). If the *spv* genes are arranged as an operon, transposon insertion mutations may cause a polar effect on the expression of downstream genes but would allow expression of the upstream genes. Insertions into *spvR*, the positive regulator gene, resulted in loss of expression of all three proteins. These data do not distinguish between loss of expression of the Spv proteins as a result of disruption of the regulator protein SpvR and loss of expression due to a possible polar effect of the transposon on downstream genes. Insertion mutagenesis of *spvA* resulted in loss of expression of SpvA, -B, and -C, and mutagenesis of *spvB* resulted in expression of SpvA but not SpvB or SpvC. These results are consistent with the model of the *spv* genes expressed from a monocistronic message. Unexpect-

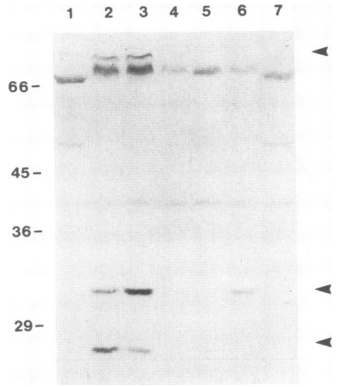

FIG. 5. Effects of transposon mutagenesis on expression of the virulence proteins. p9-18Δ7 is a deletion derivative of the native plasmid that contains all the virulence region. *spvR* and *spvABC* are arranged contiguously along the plasmid and are transcribed in the same direction. *S. dublin* LD842 (plasmid free, lane 1), Lane (wild-type plasmid, lane 2), LD842(p9-18Δ7) (lane 3), and LD842 (p9-18Δ7::Tn*1725*) containing inserts into *spvR* (lane 4), *spvA* (lane 5), *spvB* (lane 6), and *spvC* (lane 7) were grown in complete M_9 medium to the late-stationary phase and evaluated by Western blot analysis.

edly, however, mutations in *spvC* resulted in loss of expression of all three proteins. Interestingly, the same pattern of expression of the Spv proteins was found when translation termination linker insertions into *spvR*, *-A*, *-B*, and *-C* (41) (kindly provided by Donald Guiney, University of California, San Diego) were evaluated (data not shown). These oligonucleotide insertions were generated from pCR4, a plasmid containing the 8.2-kb *Xho*I-*Sal*I virulence region of pSDL2 and includes genes *spvR*, *spvA* through *-D*, and *orfE*. When protein synthesis from pCR4 was evaluated by *E. coli* minicell analysis, the translational insertion mutations were shown to be nonpolar (41).

The loss of expression of SpvA, -B, and -C with either transposon insertion or translational termination linker insertion into *spvC* indicates that the regulation of expression of these virulence proteins may be more complicated than that of a single activator protein turning on expression of downstream virulence genes. Alternatively, instability of the RNA transcript or the Spv proteins may explain loss of expression of SpvA and SpvB with insertions in *spvC*.

DISCUSSION

A number of studies have begun to examine the regulation of expression of the *spv* genes. The predicted N-terminal amino acid sequence of SpvR is homologous to those of a group of bacterial activator proteins, and several investigators have demonstrated the role of *spvR* as an activator of downstream gene(s) expression (5, 9, 25, 29, 50). Data from Taira et al. also suggested that *spvR* positively regulates its own expression (50). By primer extension analysis with an *spvA* specific probe, Krause et al. (25) identified two transcriptional start sites upstream of *spvA* after probing total RNA isolated from salmonellae grown under inducing conditions. Clear transcriptional start sites were not identified when RNA was probed with *spvB*-, *C*-, or *D*-specific probes. Additionally, these same investigators identified mono- and polycistronic mRNA transcripts by using various *spv* gene-specific probes, which were interpreted as representing *spvA*, *spvAB*, *spvABC*, and *spvABCD* transcripts. They could not exclude the possibility of *spvBC* messages comigrating with the *spvAB* transcript (25). Taken together, these data suggest that the 8.2-kb *Xho*I-*Sal*I region may contain an operon where activation or upregulation of *spvR* expression results in subsequent expression of the downstream *spvABCD* genes.

To test this model, *S. dublin* LD842 carrying a mutated plasmid derivative of pSDL2 with individual transposon insertions in *spvR* or *spvA*, *-B*, or *-C* were grown under conditions that induced expression of the Spv proteins. Expression of all three proteins was lost with disruption of *spvR*, which supports the role of SpvR as an activator. None of the Spv proteins was expressed when transposons were inserted into *spvA*, and only SpvA was expressed when insertions into *spvB* were evaluated. These results suggest that the virulence proteins may be transcribed as an operon. However, none of the Spv proteins was expressed from *S. dublin* carrying mutations in *spvC*. Similar results were obtained when the polyclonal antiserum was used to probe expression of the Spv proteins from *S. dublin*(pCR4) containing translation termination linker insertions into *spvR* or *spvA*, *-B*, or *-C* (41). Loss of expression of SpvA and SpvB with insertions into *spvC* may be a result of disruption of the stability of the transcript or may indicate that SpvC is required for expression of the other proteins. The latter explanation seems less likely, since the SpvA protein was expressed when insertions into *spvB* resulted in loss of expression of SpvC. This observation also implies that the regulation of these *spv* genes is more complex than that of a classical operon.

The pattern of Spv protein expression for the translation termination linker insertions into *spvA*, *-B*, or *-C* described above were in contrast to those reported by Roudier et al. (41). They reported that only expression of the gene containing the insertion was disrupted; neither upstream nor downstream protein expression was affected when protein expression from mutated pCR4 was evaluated in *E. coli* minicells. These differences may be due to the background of the host bacteria and suggest that chromosomal regulatory elements are operational in salmonellae but not *E. coli*.

Interestingly, Krause et al. found a hierarchy of expression of the *spv* genes, where the most abundant mRNA transcript corresponded to *spvA* followed by a progressive decrease in *spvAB*, *spvABC*, and *spvABCD* transcripts, in that order (25). Our data suggest that under the combined stresses of heat shock and glucose deprivation, this hierarchy of expression is even more pronounced and could indicate that *spvA* may not be controlled by the same promoter as the *spvB* and *spvC* genes under some conditions. Under conditions of heat shock and glucose deprivation, SpvA was strongly expressed, whereas lower levels of SpvB and SpvC were detected. The lower levels of SpvB and SpvC may be due to downregulation of expression of the *spvAB*, *spvABC*, and *spvABCD* transcripts compared with that of the *spvA* transcript or to higher susceptibility to degradation of the RNA transcripts or the proteins. It is also noteworthy that previous investigators reported that heat shock did not induce β-galactosidase activity from plasmids containing *spvA*::*lacZ* or *spvB*::*lacZ* gene fusions (9, 17). However, Gulig et al. did identify a σ^{32} consensus promoter (heat shock) upstream of the structural gene of *spvA* by DNA sequence analysis (17).

The protein products expressed from the virulence region have been identified by a number of techniques, including the use of maxicells, minicells, in vitro transcription-translation systems, high-copy-number plasmid vectors, and ex-

pression vectors (17, 20, 29, 35, 41, 49), but expression of the virulence proteins from the native plasmid harbored in wild-type *Salmonella* strains has not been reported. Progress in learning about the regulation of expression or function of these proteins has been hampered by their apparent low level of expression under standard laboratory conditions. Previous work indicates that the proteins expressed from the 8.2-kb *Xho*I-*Sal*I region are not involved with adherence, invasion, or serum resistance (19, 21, 22). The serum resistance phenotype was mapped to genes located outside of the 8.2-kb *Xho*I-*Sal*I region of the *Salmonella* plasmids (23, 52). However, this virulence region has been associated with the development of a lethal systemic infection or persistence of splenic infection in BALB/c mice. Thus, there has been speculation that the proteins expressed from the *spv* operon allow nontyphoid salmonellae to resist host defense mechanisms, possibly at the level of the macrophage (20, 22, 47). Chromosomal mutations in salmonellae that attenuate macrophage survival and also demonstrate a corresponding decrease in virulence in a mouse model (11, 12, 34) have been identified.

In fact, the intracellular milieu of a macrophage presents a particularly hostile environment. An organism must be able to (i) survive exposure to reactive oxygen radicals, lysosomal enzymes, and granular proteins such as defensins; (ii) compete with the host for nutrients such as glucose and iron; and (iii) resist acidification within the phagosome and, to a greater extent, within the phagolysosome. To gain insight into the signals that induce expression of the Spv virulence proteins from *S. dublin*, bacteria were grown in vitro under a variety of environmental conditions designed to imitate those that the organisms may encounter within the host. Polyclonal antibodies against SpvA, -B, and -C were made to augment detection of the virulence proteins.

Several stress-inducing conditions that resulted in expression of the three virulence proteins studied were identified. In all media tested and under all conditions tested except one (anaerobiosis), all three proteins were expressed when the bacteria reached the stationary phase. These results agree with the pattern of expression of β-galactosidase activity from stationary-phase salmonellae containing *spv*::*lacZ* gene fusions reported previously (5, 9, 25). Both of these groups of investigators used the 8-kb virulence region or fragments of this region cloned onto a multicopy vector to examine the expression of β-galactosidase activity from *lacZ* fusions, and thus expression was not evaluated from the native plasmid (5, 9, 25). However, all of these studies suggest that the Spv proteins are expressed minimally or not at all under noninducing conditions and that they are then turned on maximally in the presence of the appropriate signal.

KatF encodes a putative sigma factor that regulates genes expressed during starvation conditions, and Gulig et al. identified KatF-regulated promoter consensus sequences upstream of *spvR* and *spvC* (17). *katF* was recently mapped on the *Salmonella* chromosome by Fang et al., who also reported that growth of *S. typhimurium katF* mutants under *spv* inducing conditions resulted in decreased *spvB* expression (10). Entry into the stationary phase of growth, however, is not a prerequisite for induction of the Spv proteins; when *S. dublin* Lane was grown in complete medium with only Casamino Acids as a carbon source, the proteins were detected in early-exponential growth. Interestingly, Schellhorn and Stones recently reported that spent culture supernatants recovered from *E. coli* in the stationary phase and weak acids such as acetate induced expression of *katF* in cells in the exponential phase (44). These results suggest that metabolic products accumulating in stationary-phase cultures may be responsible for the induction of the Spv proteins; these metabolites may accumulate in exponentially growing cells if Casamino Acids are provided as the sole carbon source. Further studies are needed to determine which metabolite is responsible for induction of the Spv proteins. Stationary-phase growth may not in itself be an adequate signal for expression of Spv proteins. Salmonellae grown anaerobically to the stationary phase never expressed these proteins. Alternatively, these data may be interpreted as a requirement for oxygen for expression of the Spv proteins. In addition to stationary growth and glucose starvation, the SpvA, -B, and -C plasmid virulence proteins of *S. dublin* Lane were expressed from the native plasmid in vitro under iron limitation, acidification, and growth at 42°C.

All of these stress-inducing conditions may be encountered by salmonellae in the host, particularly within the intracellular milieu of the macrophage. Salmonellae are equipped to respond to a variety of stressful encounters by induction and suppression of distinct subsets of proteins. Characteristic protein responses have been described for bacteria subjected to such stressful stimuli as limitation of carbon sources (stringent response), increased temperatures (heat shock response), DNA damage (SOS response), exposure to reactive oxygen intermediates (oxidative stress response), and decreased pH (acid tolerance response). Buchmeier and Heffron recently reported that infection of macrophages with salmonellae also leads to the selective synthesis of over 30 *Salmonella* proteins (4), which may be considered an intracellular stress response. While much remains to be learned about the relatedness and regulation of expression of the multiple stress responses, it has long been recognized that different types of stress can produce overlapping if not identical responses. For example, the so-called heat shock response proteins can also be induced by oxidative stress, starvation, and UV irradiation. Foster and Hall recently reported a relationship between iron- and pH-regulated protein synthesis in *S. typhimurium* (14). It is quite possible that the Spv virulence proteins may be under the regulation of one or more of these protective stress responses.

Generation of polyclonal antibodies allowed detection of the SpvA, -B, and -C virulence proteins from the native plasmid in vitro for the first time. These antibodies will be useful in determining whether the interaction of the bacteria with tissue culture cells or with BALB/c mice during the course of a natural infection will also induce expression of the virulence proteins. Understanding where and when in the host that these proteins are expressed may provide clues about their function.

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